



Anti-inflammatory, Analgesic and Antipyretic Properties of Ethanolic Extracts of Three Plants of Beninese's Pharmacopoeia: *Euphorbia hirta*, *Citrus aurantifolia* and *Heterotis rotundifolia*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OGR, VD and LBM designed the project and supervised the work. Authors OGR and JRK carry out the experiments, analyzed and interpreted the data. Authors OGR, VD and AH wrote the manuscript. Authors VD and LBM edited the manuscript. All authors read and approved this manuscript.

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ABSTRACT

Background/Objective: *Euphorbia hirta*, *Citrus aurantifolia* and *Heterotis rotundifolia* are commonly used in Benin in the treatment of infectious diseases. The aim of this study is to evaluate the anti-inflammatory, analgesic and antipyretic properties of ethanolic extracts of these plants.

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Materials and Methods: The study was carried out on 30 wistar rats placed in 6 work lots. A positive control lot having received diclofenac and a negative control lot having received physiological water were used. The ethanolic extract of the plants was used at a dose of 200 mg / kg bw. The model of inflammatory edema of the rat paw induced by 2% formalin was used. Analgesic activity was assessed by the pain method induced by 3% acetic acid and the tail immersion method with wistar rats. The antipyretic effect was evaluated on pyrexia induced by brewer's yeast at 20% with wistar rats.

Results: After injection of formalin to animals, inflammatory reaction was almost immediate with appearance of classic signs of acute local inflammation (Redness, pain, heat and edema) at the five experimental groups. This inflammatory reaction occurs in two phases. The first phase occurs between 0 and 2 hours after injection of phlogogenic agent and the second phase, initiated after two hours extending to the fifth hour and even beyond. Administration of these extracts prevents edema inflammatory and inhibition percentages of edema vary between 23.67% and 86.76% for the three extracts. These extracts have similar anti-inflammatory activity ($p > 0.05$) to that of diclofenac at 50 mg/kg. Analgesic activity show that these extracts inhibit very significantly ($p < 0.001$) chemical pain induced by acetic acid and the highest inhibition percentage is 60.34% (*Citrus aurantifolia*). This percentage is like to that of acetylsalicylic acid (67.35%) administered at the same dose. Likewise, these extracts attenuate significantly ($p < 0.05$) thermal pain induced by tail immersion of each rat in hot water at 50°C. Indeed, these extracts, reduces very significantly ($p < 0.001$) pyrexia induced by 20% beer yeast suspension in rats and they have similar effect ($p > 0.05$) to that of acetylsalicylic acid at the fourth hour.

Conclusion: These results show that the plants studied have the pharmacological properties evaluated. These results justify the use of these plants in traditional medicine.

Keywords: Treatment; pharmacopoeia; *Euphorbia hirta*; *Citrus aurantifolia*; *Heterotis rotundifolia*; Benin.

ABBREVIATIONS

EE-Eh : Ethanolic Extracts of *Euphorbia hirta*
 EE-Ca : Ethanolic Extracts of *Citrus aurantifolia*
 EE-Hr : Ethanolic Extracts of *Heterotis rotundifolia*
 b.w : body weight
 EAP : Edema Increase Percent
 EIP : Edema Inhibition Percent
 Dt : Mean diameter of the right hind paw at time t
 Do : Mean diameter of the right hind paw at time 0 (before treatment)
 ASA : Acetylsalicylic Acid
 CIP : Cramping Inhibition Percent
 TCc : Mean of twisting count of control group
 TCt : Mean of twisting count of treated groups
 PIP : Pyrexia Inhibition Percent
 T°o : Temperature before pyrexia induction
 T°n : Temperature after pyrexia induction and treatment at time t
 NSAIDs : Nonsteroidal Anti-inflammatory Drugs

1. INTRODUCTION

Inflammation is a reaction implemented by an organism whenever its morphological and

biological constants are threatened [1]. It may have an infectious origin or caused by physical or chemical agent [2]. Usually, inflammatory response is a beneficial process. Its purpose is to eliminate the pathogen and repair tissue damage. However, inflammation can be harmful, because of the aggressiveness of the pathogen, its persistence, by abnormal regulation of the inflammatory process, or by quantitative or qualitative abnormalities of cells involved in inflammation [3]. It can be manifested by various symptoms such as edema, pain and heat usually accompanied by fever.

Nowadays, anti-inflammatory and/or analgesic potential molecules are used in the treatment of inflammatory diseases. They are steroidal and non-steroidal anti-inflammatory drugs [4,5]. These molecules, despite their efficiencies, most often have side effects, which can cause other annoyances as a result of prolonged use [6,7]. Indeed, the use of phytochemicals compound is useful and without side effects [8]. Besides, herbal medicine has been used for centuries to treat several diseases. Thus, herbal teas, decoctions, macerations, infusions and plasters are used successfully [9].

In the rich and diverse Benin flora, *Euphorbia hirta*, *Citrus aurantifolia*, and *Heterotis rotundifolia* are known and used as having effects on conditions that cause inflammatory reactions [10,11]. Especially that inflammation and pain are a common feature of many diseases [12]. Due to the wide use of these plants in traditional medicine, their extracts have been the subject of several phytochemical studies. The richness of these plants in polyphenols and flavonoids gives them several pharmacological activities [13]. Nevertheless, very few studies have focused on their anti-inflammatory activity. That's why this study has the aim to evaluate anti-inflammatory, analgesic and antipyretic properties of the ethanolic extracts of these plants (*Euphorbia hirta*, *Citrus aurantifolia*, *Heterotis rotundifolia*). The results of this study can be used to enhance and improve their use.

2. MATERIALS AND METHODS

2.1 Animals Used

30 Male and female Wistar rats having 10 to 12 weeks old and weighing between 150 and 200 g were used. These animals came from the Applied Biomedical Sciences Institute of University of Abomey-Calavi. These animals were acclimatized to the conditions of the animal house for a week before experiments. They were fed from the standard pellets with access to water at will. The cycle of light was 12/24h.

2.2 Plants Used

The whole plants of *Euphorbia hirta* (*E. hirta*) and *Heterotis rotundifolia* (*H. rotundifolia*) and the leaves of *Citrus aurantifolia* (*C. aurantifolia*) were used. They were harvested (At Kpomassè for *E. hirta* and Abomey-Calavi for *C. aurantifolia* and *H. rotundifolia*) and confirmed at the national herbarium of University of Abomey-Calavi. These plants were dried in the laboratory at 25°C for two weeks. After drying, they were powdered in laboratory. The powders were used to make ethanolic extracts.

2.3 Ethanolic Extracts Preparation

The ethanolic extracts are obtained by maceration of 50 g of powder of each plant in 500 ml of 96% ethanol for 48 h with continuous stirring. The mixture was then filtered twice on hydrophilic cotton and once on N°1 Whatman

paper before being concentrated in a rotary evaporator at 50°C. The concentrates were deposited in an oven at 50°C until obtained a dry mass which constitutes the ethanolic extract [14].

2.4 In vivo Anti-inflammatory Activity of Extracts

The method used here has been inspired by that of Sy et al. [15] with some modifications. Thus, 5 groups of 6 rats each were formed according to the weight and fasted 15 hours before experimentation. The treatment was done orally as follows:

- The first animals group were used as controls and they received only physiological water at 1 ml/100 g body weight (b.w.);
- The second animals group were used as a reference and they received diclofenac at 50 mg/kg b.w.;
- The other three animal's groups received ethanolic extracts of *E. hirta* (EE-Eh), *C. aurantifolia* (EE-Ca) and *H. rotundifolia* (EE-Hr) at 200 mg/kg b.w. respectively.

One hour after treatments, 0.1 ml of 2% formalin solution was injected into each rat under foot pad of the right hind paw. The paw diameter at the arch was measured using electronic display calipers every hour until fifth hour. Edema increase (EAP) and inhibition (EIP) percentages were calculated according to the formula:

$$EAP = \frac{Dt - Do}{Dt} \times 100$$

Where Dt: Mean diameter of the right hind paw at time t; Do: Mean diameter of the right hind paw at time 0 (before treatment).

$$EIP = \frac{(EAP)_{control\ group} - (EAP)_{treated\ group}}{(EAP)_{control\ group}} \times 100$$

2.5 Analgesic Activity of Extracts

2.5.1 Writhing test

The method described by Koster et al. [16] and taken over by Sy et al. [15] was used. Thus, 5 groups of 6 rats each were formed and treated in the same way as before. But the reference animals group used here received acetylsalicylic acid (ASA) at 200 mg/kg b.w. Thirty minutes after treatments, 0.1 ml of 3% acetic acid solution was injected intraperitoneally to all rats and the

twisting for each rat was counted over 30 minutes. Cramping inhibition percentage (CIP) was calculated according to the formula:

$$CIP = \frac{TCc - TCt}{TCc} \times 100$$

Where TCc represents the average of the number of twists of the control group (group not treated with the plant extract) and TCt represents the average of the number of twists of the groups treated with the plant extract.

2.5.2 Tail immersion method

To evaluate analgesic effect of extracts by this approach, the method described by Gbenou et al. [17] was used with some modifications. The rats were arranged and treated as in the writhing test. Thirty minutes after treatments, the tail of each animal was immersed in hot water at 50°C and the reaction time was recorded.

2.6 Antipyretic Effect of Extracts

Pyrexia was induced by subcutaneous injection in 5 groups of 6 rats each with a 20% beer yeast suspension. 24 hours after this induction, rats that showed an increase in temperature were treated with extracts and ASA as described above. The anal temperature of each rat was recorded using an electronic display thermometer every hour for 4 hours [18]. Pyrexia inhibition percentage (PIP) was calculated according to the formula:

$$PIP = \frac{(T^{\circ}t - T^{\circ}o)_{control\ group} - (T^{\circ}t - T^{\circ}o)_{treated\ group}}{(T^{\circ}t - T^{\circ}o)_{control\ group}} \times 100$$

Where T[°]o: Temperature before pyrexia induction; T[°]t: Temperature after pyrexia induction and treatment at time t

Indeed, before this test, anal temperature of each rat was recorded morning, noon and evening for 3 days and alone the rats which showed a stability of internal temperature have been used.

Otherwise, it should be noted that the choice of 200 mg/kg b.w. as a dose for all tests, is due to the fact that the extracts may not act quickly at a low dose because they are not pure molecules. However, evaluations are carried out in a time interval. Thus, in previous study, a toxicological evaluation of these extracts was performed with wistar rats at a limit dose of 2000 mg/kg b.w. and we noted that these extracts do not have any toxicity and any influence on hematological and biochemical parameters [19-21]. This is why, we

have made a 10% reduction of this dose to evaluate in vivo the anti-inflammatory, analgesic and antipyretic activities of these extracts.

2.7 Statistical Data Processing

GraphPad Prism 7 software was used to perform graphs and statistical analyzes. A comparison of mean was made with analysis of variances (ANOVA two ways) followed by Tukey's multiple comparison test. The differences are considered significant if p-value < 0.05 and very significant if p-value < 0.001.

3. RESULTS

Fig. 1 show, percentages of edema increase (a) and inhibition (b) of right hind paw of rats. The extracts prevent very significantly (p<0.001) from third to fifth hour edema in rats compared to control. This prevention is similar to that of rats treated with diclofenac at the same time intervals. The increase percentages of edema are steady (between 45.72 and 49.95%) in control rats, whereas in treated rats (diclofenac and extracts), these percentages decrease from the first to the fifth hour (Fig. 1a). We also note that the inhibition percentages of edema increase with time and there is no significant difference (p>0.05) between the anti-inflammatory effect of extracts at 200 mg/kg and that of diclofenac at 50 mg/kg from first to fifth hour. However, the highest inhibition percentages of inflammatory edema were observed at the fifth hour in all treated rats. So we have: 79.80%, 86.75%, 83.07% and 87.94% respectively for *E. hirta*, *C. aurantifolia*, *H. rotundifolia* and diclofenac (Fig. 1b).

Table 1 and Fig. 2 show analgesic activity of extracts. The administration of ASA and extracts has very significantly (p < 0.001) prevented pain to the rats. Which's noted by a clear decrease twisting count in treated rats (Extracts and ASA). Inhibition percentages ranges from 35.39% to 60.34% in extracts-treated rats (Table 1). The reaction time is 4.66 seconds in control rats after immersion of their tail in hot water at 50°C. This time has significantly (p<0,05) increased and ranges from 10.66 to 14 seconds in rats treated with extracts (Fig. 2).

Fig. 3 show, the evolution of temperature of control and treated rats (a) and pyrexia inhibition percentages (b). In control rats, the temperature remained high (between 38.43°C and 39.06°C) while in treated rats (ASA and extracts) there was a decrease until the fourth hour when it was

Table 1. Effect of extracts and ASA on pain induced by 3% acetic acid in rats

	Control	ASA	EE-Eh	EE-Ca	EE-Hr
Twisting Count	41.5±3.44	13.5±4.4**	23 ±3.79**	16.33 ±3.32**	26.67±4.88**
CIP (%)	--	67,35±11,23 ^{ns}	43.97±11.76	60,34±9.01 ^{ns}	35.39±13.06

Mean±SE Diegends: EE-Eh: Ethanolic Extracts of Euphorbia hirta; EE-Ca: Ethanolic Extracts of Citrus aurantifolia; EE-Hr: Ethanolic Extracts of Heterotis rotundifolia; ASA: Acetylsalicylic Acid; CIP: Cramping Inhibition Percentages; With **: very significant difference with Control; With ns: no significant difference between them

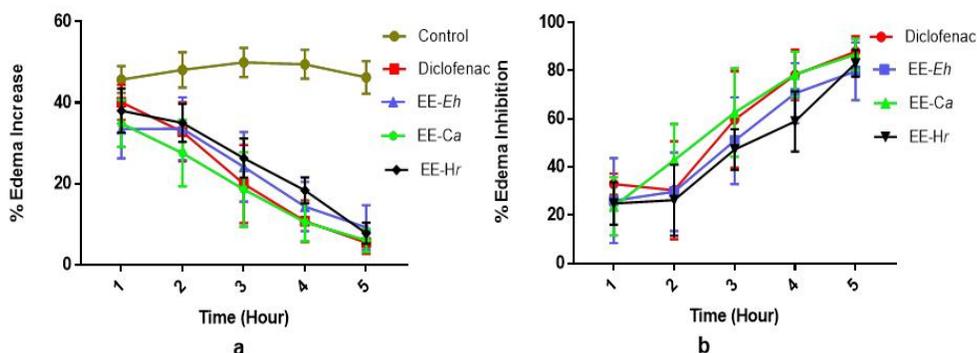


Fig. 1. Percentages of oedema increase (a) and inhibition (b) of right hind paw of rats
 Fig. 1 legends: EE-Eh: Ethanolic Extracts of Euphorbia hirta; EE-Ca: Ethanolic Extracts of Citrus aurantifolia; EE-Hr: Ethanolic Extracts of Heterotis rotundifolia; a: Percents of Edema Increase; b: Percents of Edema Inhibition

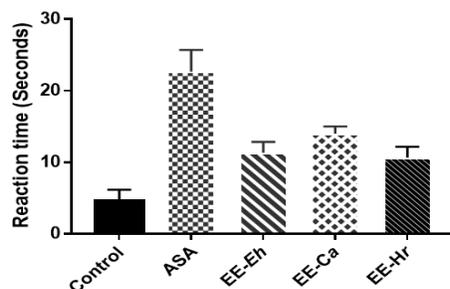


Fig. 2. Reaction time of control and treated rats after tail immersion in hot water at 50°C
 Fig. 2. legends: EE-Eh: Ethanolic Extracts of Euphorbia hirta; EE-Ca: Ethanolic Extracts of Citrus aurantifolia; EE-Hr: Ethanolic Extracts of Heterotis rotundifolia; ASA: Acetylsalicylic Acid

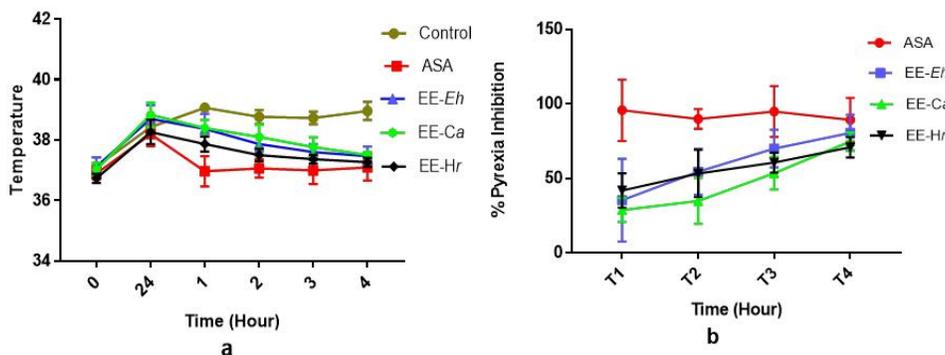


Fig. 3. Evolution of temperature of control and treated rats (a) and Pyrexia inhibition percentages (b)

Fig. 3. legends: EE-Eh: Ethanolic Extracts of Euphorbia hirta; EE-Ca: Ethanolic Extracts of Citrus aurantifolia; EE-Hr: Ethanolic Extracts of Heterotis rotundifolia; ASA: Acetylsalicylic Acid; a: Evolution of temperature of control and treated rats; b: percents of pyrexia inhibition

very significant ($p < 0.001$) compared to the control (Fig. 3a). Indeed, pyrexia inhibition percentages vary between 70.89% and 80.61% for extracts-treated rats at fourth hour. These percentages are similar ($p > 0.05$) to that of the ASA at the same hour (Fig. 3b).

4. DISCUSSION

The extracts, at 200 mg/kg, have edema inhibition percentages similar ($p > 0.05$) to that of the standard (diclofenac) in the second phase of the inflammatory process (Fig. 1). Many studies have reported that diclofenac acts at the 2nd phase of inflammation, by inhibiting prostaglandins releasing through cyclooxygenase (COX2) inhibition [22, 23]. It can therefore be deduced that the extracts at 200 mg/kg would have acted in the same way as the diclofenac. Indeed, the anti-inflammatory activity of plants is often attributed to secondary metabolites. Dougnon et al. [13] had revealed polyphenols and flavonoids presence in the ethanolic extracts of plants studied. In addition, several studies have already shown the anti-inflammatory properties of bioactive molecules belonging to the flavonoid family [24,25,26].

The highest edema inhibition percentages of extracts of these plants are 79.80% (EE-Eh), 86.75% (EE-Ca) and 83.07% (EE-Hr) obtained at fifth hour. These percentages are greater than 67.17% obtained with aqueous extract of *Elaeis guineensis* at 500ml/kg in Sene et al. [27] studies. On the other hand, they are less than 98% and 90% obtained respectively with aqueous extracts of *Sterculia setigera* and the mixture *Aframomum melegueta* - *Citrus aurantifolia* at 1500 mg/kg in Gbénou et al. [17] studies. The difference observed between results of this study and those of aforementioned authors, can be explained by the difference at the plants and extracts used as well as the difference in assessed doses. 3% acetic acid injection causes chemical pain in rats, which is manifested by abdominal cramps and twists, through chemical mediators releasing such as bradykinin, serotonin, acetylcholine and prostaglandins. The fact that the extracts inhibits very significantly ($p < 0.001$) this chemical pain such as ASA at 200 mg/kg suggests that these extracts may interfere with these mediators like salicylates. Same observations were made in Uche et al. [28] studies.

Furthermore, animals tail immersion in hot water at 50°C causes tail sudden movement and sometimes an overall animals body retreat in

control rats. There was a significant ($p < 0.05$) and very significant ($p < 0.001$) increase in reaction time, respectively in extracts-treated and standard-treated rats (Fig. 2). The reaction time varies between 10.66 (EE-Hr) and 14 seconds (EE-Ca) in extracts-treated rats. These times are greater than 4.47 ± 0.19 seconds and 5.48 ± 0.27 seconds obtained by Bose et al. [29] with *Cleome rutidosperma* ethanolic extract at 200 mg/kg and 400 mg/kg respectively. The inhibition of this type of pain by plants studied extracts, had already been demonstrated with *Paederia sandens* extracts [30].

Indeed, the same mediators that cause inflammation and pain often cause fever. This is why antipyretic effect of these extracts has been assessed. The results show that the extracts oppose fever installation by gradually lowering the temperature in extracts-treated rats. Similar result was obtained with ASA with faster effect. It's therefore possible that extracts inhibits prostaglandins synthesis like ASA [31]. Antipyretic effect observed in this study is similar to that observed by Morabandza et al. [18] in their study with aqueous extracts of stem bark of *Strychnos camptoneura* at different doses.

5. CONCLUSION

E. hirta, *C. aurantifolia* and *H. rotundifolia* at 200 mg/kg b.w., have anti-inflammatory, analgesic and antipyretic effects similar to nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac and acetylsalicylic acid. The simultaneous presence of these biological activities in these extracts could be explained by the presence of flavonoids known for their inhibitory activity of prostaglandins that generate these pathological conditions.

Our results confirm and justify the traditional use of these plants in the treatment of various diseases.

6. LIMITATIONS

The current study only investigated *in vivo* anti-inflammatory, analgesic and antipyretic effects of ethanolic extracts of *E. hirta*, *C. aurantifolia* and *H. rotundifolia*. It would be better to complete this study by measuring the mediators (histamine, prostaglandin, interleukins....) involved in these pathologies (before treatments and after observations) and to explore the mechanism of action of these extracts.

CONSENT

It is not Applicable.

ETHICAL APPROVAL

All the experiments were conducted according to the protocol approved by the ethics committee of the Research Unit in Applied Microbiology and Pharmacology of natural substances-University of Abomey-Calavi (URMAPha-UAC) under the number 037-19/URMAPHA/EPAC/UAC. In addition, all experimentation was carried out in accordance with Act No. 2010-40 of December 08, 2010 on the code of ethics and deontology for health research in the Republic of Benin.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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